



## The Safety Evaluation of a Potent Angiogenic Activator, Synthetic Peptide (SFKLRV-NH<sub>2</sub>) for the Skin Application

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A novel synthetic hexapeptide (SFKLRV-NH<sub>2</sub>) that displays angiogenic activity has been identified by positional scanning of a synthetic peptide combinatorial library (PS-SPCL). This study was carried out to investigate the irritation of the SFKLRV-NH<sub>2</sub> on the skin. The tests were performed on the basis of Korea Food and Drug Administration (KFDA) guidelines. In results, cell toxicity is not appeared for SFKLRV-NH<sub>2</sub> in HaCaT cells and B16F10 cells. SFKLRV-NH<sub>2</sub> induced no skin irritation at low concentration (10 μM), mild irritation at high concentration (10mM). We consider that this result is helpful for saying about the safety of SFKLRV-NH<sub>2</sub> in clinical use.

**Key words:** PS-SPCL, Safety, Skin irritation test, SFKLRV-NH<sub>2</sub>, W3 peptide

### INTRODUCTION

The one of the physiological response of the exposition and accidental contact with new chemical entities is irritation to skin, which involves local redness and edema. Thus, assessment of skin irritation potential is an important part of preclinical safety assessment for new chemical entities before humans can be exposed to such substances. Draize skin irritancy tests have been the standard for prediction of human dermal irritation for decades since Draize's techniques were used by the Food and Drug Administration to evaluate the safety of several substances.

SFKLRV-NH<sub>2</sub> (Ser-Phe-Lys-Leu-Arg-Tyr-NH<sub>2</sub>, W3 peptide®, NovaCell Technology Inc, Pohang, Korea), prototype peptide was proved to be able to induce DNA synthesis, migration, and tube formation through PTX-sensitive-G protein/PLC mediated [Ca<sup>2+</sup>]<sub>i</sub> increase in endothelial cells, which are the essential steps for angiogenesis (Lee *et al.*, 2009).

Identification of SFKLRV-NH<sub>2</sub> is noticeable in angiogenesis studies as a small peptide. SFKLRV-NH<sub>2</sub> showed angiogenic activity not only in vitro and ex vivo but also had dramatic wound healing activity in rat model, thus, we suggest that our peptide could be regarded as a good candidate for the treatment against the wound healing and such condition where the formation of new blood vessel is needed (Lee *et al.*, 2009). This new chemical entities, identified a novel hexapeptide inducing [Ca<sup>2+</sup>]<sub>i</sub> increase in endothelial cells by accommodating positional scanning method of synthetic peptide combinatorial library (PS-SPCL) (Houghten *et al.*, 1991; Lee *et al.*, 2009).

The following guidelines present an alternative, by using human volunteers, to the patch test for assessment of the skin irritation potential of cosmetic-finished products. As irritancy is minimal, the 'skin compatibility' to humans is assessed. Skin compatibility is defined as the absence of skin irritation under normal conditions of use and reasonably foreseeable misuse, taking into account objective reactions as well as subjective responses such as stinging, burning or itching (Aoshima *et al.*, 2009).

In principle, the aim is to assess the compatibility of test materials to the skin in studies carried out ethically on human volunteers. The goal can be achieved by using a cautious stepwise approach adapted to these materials to be tested. The aim of present study was therefore to investi-

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gate the tendency of SFKLRV-NH<sub>2</sub> to cause skin irritation. Thus, we carried out skin irritation test on human volunteers. The tests were performed on the basis of Korea Food and Drug Administration (KFDA) guidelines (KFDA, 2009). Overall the data indicate that SFKLRV-NH<sub>2</sub> is likely to be no irritant to skin.

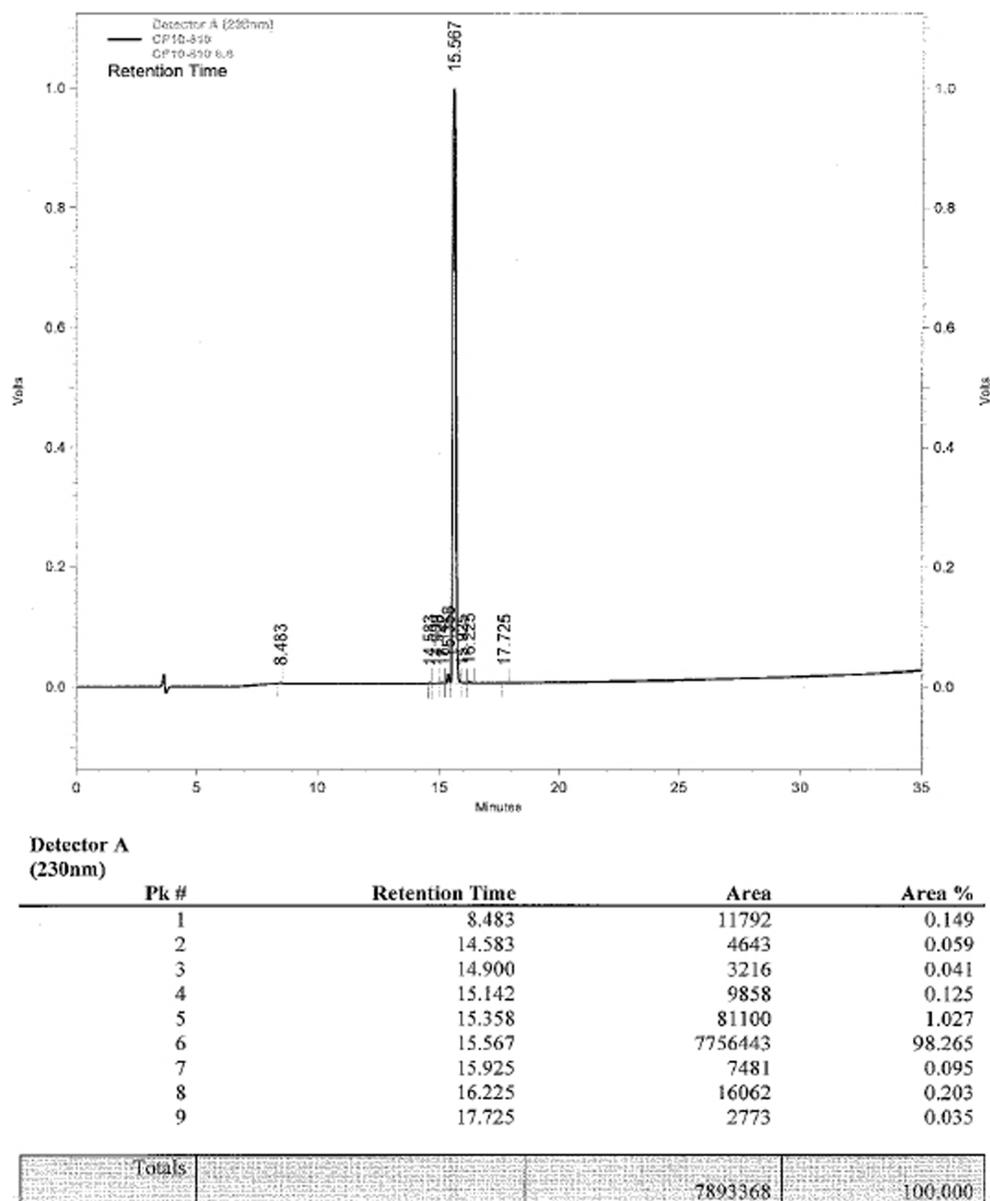
## MATERIALS AND METHODS

**Materials.** SFKLRV-NH<sub>2</sub>, a prototype peptide were synthesized by Pepton Inc. (Korea). PS-SPCLs were prepared in the Peptide Library Support Facility at Pohang University of Science and Technology (Korea), as previously

described (Baek *et al.*, 1996; Houghten *et al.*, 1991). The purity of SFKLRV-NH<sub>2</sub> was determined at 98.1% by using of high performance liquid chromatography (Fig. 1).

Dulbecco's modified Eagle's medium (DMEM), Ham's F-12, fetal bovine serum (FBS, Hyclone, Logan, UT, USA), and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Gibco Ltd. (Grand Island, NY, USA).

**Cell cultures.** B16 murine melanoma cells and Human HaCaT keratinocytes were obtained from the Korean Cell Line Bank (Seoul). Cells were incubated in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 50 µg/ml streptomycin and 50 µg/ml penicillin at 37°C in 5% CO<sub>2</sub>.



**Fig. 1.** A purity of SFKLRV-NH<sub>2</sub> in high performance liquid chromatography.

**Determination of cell viability.** The B16F10 cells were seeded in a 6 well plate at a density of  $1 \times 10^5$  cells per well. And HaCaT cells were seeded in a 12 well plate ( $2 \times 10^5$  cells per well). After overnight, various concentrations of SFKLRY-NH<sub>2</sub> range from 0.01  $\mu$ M to 10  $\mu$ M were treated in 6 well or 12 well plate. The each treated plate was incubated in 37°C CO<sub>2</sub> incubator for 24 hr. The percentage of viable cells was determined by staining the cells with MTT (Mosmann, 1983). The culture medium was removed from each well, and the cells were washed with phosphate-buffered saline (PBS) and treated with the staining solution as MTT. The well was incubated in 37°C CO<sub>2</sub> incubator for 3 hr and removed. After adding 1 ml DMSO, the MTT absorption was measured at 570 nm.

**Clinical patch test.** The patch test was performed on the skin of 8 men and 24 women, aged 21 to 49 years with Fitzpatrick skin type III to IV (Diffey, 1991). Individuals were excluded if they had any active or history of skin diseases or conditions that may interfere with the evaluation of skin reactions, had any known allergy to skin care products, or routinely received any anti-inflammatory medications. All participants were required to sign an informed consent agreement.

The test samples (10  $\mu$ M, 10 mM) was placed on a Finn Chamber (Chemotechnique Diagnostics, Sweden) and applied to the ventral side of each subject's upper arm for 24 hour in an occlusive condition. Skin reactions were evaluated 1 and 24 hour after removing the test samples. The reaction was evaluated according to the International Contact Dermatitis Research Group (ISDRG) standard (Table 1) (Walker *et al.*, 1996; York *et al.*, 1996).

**Skin irritation test.** The test procedure was done on the "Guidelines for safety test of the drugs" provided by KFSA (KFSA, 2009). The degree of dermal irritation of SFKLRY-NH<sub>2</sub> was determined in human beings using the occluded dermal irritation test method as described elsewhere. Skin irritation test were examined for the presence of erythema and edema according to the dermal irritation scoring system (0, no erythema or no edema; 1, barely perceptible erythema or edema; 2, well defined erythema or slight edema; 3, moderate to severe erythema or moderate edema; 4, severe erythema or edema) at grading intervals of 1 hour and 24 hour.

The primary irritation index (PII) was calculated by

**Table 2.** Irritation rating for primary dermal reaction on the skin after application of SFKLRY-NH<sub>2</sub>

Ranging of P.I.I.*	Rating
P.I.I = 0	No irritated
0 < P.I.I ≤ 2	Mild irritated
2 < P.I.I ≤ 5	Moderate irritated
5 < P.I.I	Severe irritated

\*P.I.I: Primary Irritation Index.

dividing the sum of erythema and edema scores of the grading intervals multiplying by the number of grading intervals. The material was then classified according to the Draize method of classification using the PII scoring as mildly irritant (PII ≤ 2), moderately irritant (2 < PII ≤ 5), and severe irritant (PII > 5) (Table 2) (Shara *et al.*, 2005).

**Statistical analysis.** The data are represented as mean ± S.D. Statistical comparisons between groups were performed using Sigma Plot followed by Student's t-test. Data on irritation are presented as visual scores based on Draize method of erythema and edema-grading system and PII was calculated.

## RESULTS AND DISCUSSION

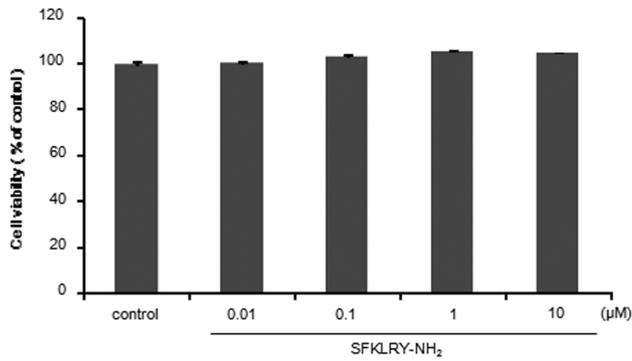
This new chemical entities, identified a novel hexapeptide inducing [Ca<sup>2+</sup>]<sub>i</sub> increase in endothelial cells by accommodating PS-SPCL. The strategy of PS-SPCL has been used to isolate peptides that have angiogenic potential (Houghten *et al.*, 1991). Moreover, the PS-SPCL method has successfully been applied to screen useful peptides that are involved in various biological processes, resulting in the identification of several peptides such as interleukin-8-specific antagonists (Hayashi *et al.*, 1995), inhibitor for nuclear factor of activated T cells (Aramburu *et al.*, 1999), and the immunomodulatory peptides (Bae *et al.*, 2001).

First, we performed the cell viability test for SFKLRY-NH<sub>2</sub> in HaCaT cells and B16F10 cells. After various concentrations of SFKLRY-NH<sub>2</sub> were treated to cells, cell toxicity means that decreasing cell number is greater than or equal to 20 percent. Cell toxicity is not appeared for SFKLRY-NH<sub>2</sub> in HaCaT cells (Fig. 2) and B16F10 cells (Fig. 3).

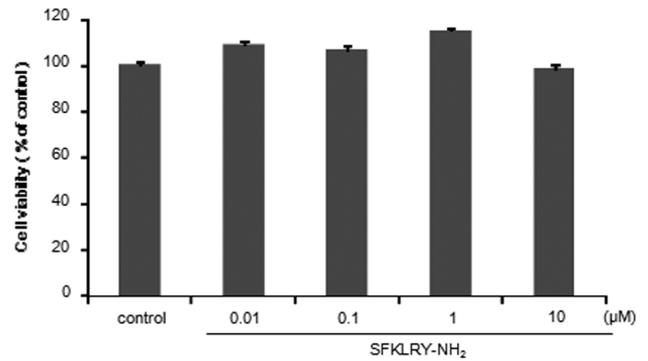
After, we carried out the skin irritation test on the 32 human volunteers (Table 3). Edema was not evident, but in the high concentration (10 mM), only one patient appeared

**Table 1.** Assessment of patch test reactions

Grading	Description of response
0	No reaction
+	Weakly positive reaction (usually characterized by mild erythema or dryness across most of the treatment site)
++	Moderately positive reaction (usually distinct erythema possibly spreading beyond the treatment site)
+++	Strongly positive reaction (strong, often spreading erythema with oedema)



**Fig. 2.** Cell viability for SFKLRN-NH<sub>2</sub> in HaCaT cells.



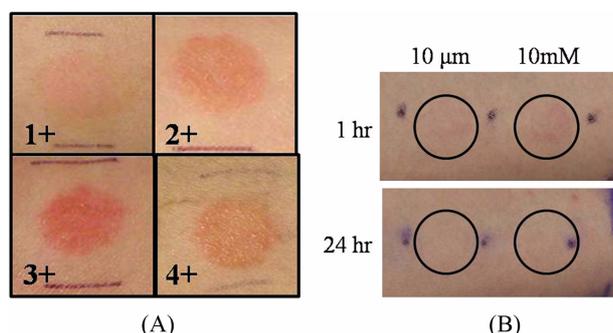
**Fig. 3.** Cell viability for SFKLRN-NH<sub>2</sub> in B16F10 cells.

**Table 3.** Results of skin reactions applied with SFKLRN-NH<sub>2</sub>

Groups			SFKLRN-NH <sub>2</sub> 10 µM				SFKLRN-NH <sub>2</sub> 10 mM			
Change			Erythema		Edema		Erythema		Edema	
Time			1 hr	24 hr	1 hr	24 hr	1 hr	24 hr	1 hr	24 hr
Number of patients	Sex	Age								
1	F	39	0	0	0	0	0	0	0	0
2	F	49	0	0	0	0	1	0	0	0
3	F	22	0	0	0	0	0	0	0	0
4	M	38	0	0	0	0	0	0	0	0
5	F	38	0	0	0	0	0	0	0	0
6	F	38	0	0	0	0	0	0	0	0
7	F	26	0	0	0	0	0	0	0	0
8	M	23	0	0	0	0	0	0	0	0
9	F	35	0	0	0	0	0	0	0	0
10	F	40	0	0	0	0	0	0	0	0
11	M	35	0	0	0	0	0	0	0	0
12	F	21	0	0	0	0	0	0	0	0
13	F	38	0	0	0	0	0	0	0	0
14	M	31	0	0	0	0	0	0	0	0
15	F	33	0	0	0	0	0	0	0	0
16	F	31	0	0	0	0	0	0	0	0
17	M	27	0	0	0	0	0	0	0	0
18	M	36	0	0	0	0	0	0	0	0
19	F	21	0	0	0	0	0	0	0	0
20	F	24	0	0	0	0	0	0	0	0
21	F	34	0	0	0	0	0	0	0	0
22	F	22	0	0	0	0	0	0	0	0
23	F	24	0	0	0	0	0	0	0	0
24	M	24	0	0	0	0	0	0	0	0
25	F	42	0	0	0	0	0	0	0	0
26	F	43	0	0	0	0	0	0	0	0
27	F	25	0	0	0	0	0	0	0	0
28	F	20	0	0	0	0	0	0	0	0
29	F	28	0	0	0	0	0	0	0	0
30	M	30	0	0	0	0	0	0	0	0
31	F	25	0	0	0	0	0	0	0	0
32	F	22	0	0	0	0	0	0	0	0
Total score			0	0	0	0	1	0	0	0
Mean score			0	0	0	0	0.03	0	0	0
P.I.I.*			0				0.01			

Erythema was scored as follows: no erythema = 0, very slight erythema (barely perceptible) = 1, well-defined erythema = 2, moderate to severe erythema = 3, severe erythema (beet redness) to slight eschar formation (injuries in depth) = 4. Edema formation was scored as follows: no edema = 0, very slight edema (barely perceptible) = 1, slight edema (edges of area well-defined by definite raising) = 2, moderate edema (raised approximately 1 mm) = 3, severe edema (raised more than 1 mm and extending beyond area of exposure) = 4.

\*Primary irritation index = total score/2.



**Fig. 4.** Clinical signs in the skin applied with SFKLRY-NH<sub>2</sub>. (A) Clinical standard photograph. Grade 1: Slight erythema, either spotty or diffuse, Grade 2: Moderate uniform erythema, Grade 3: Intense erythema with edema, Grade 4: Intense erythema with edema & vesicles. (B) Clinical signs of patient No. 2 in the skin applied with SFKLRY-NH<sub>2</sub>. One hour after detachment, slight erythema observed in the 10 μM group. But, 24 hour after detachment, there was no erythema and edema.

to have slight and slightly barely perceptible erythema effects at 1 hour, as the PII was calculated to be 0.016. There was almost no erythema persisting 24 h after opening the patch. The time course of recovery from irritation is depicted in Fig. 4. Erythema faded away quickly and the skin was back to normal within a short period. There was no erythema or edema observed on the low concentration (10 μM). Scores of erythema following 1 hour and 24 hour after opening the occlusion were zero in intensity.

Erythema decreased quickly after opening the occlusion, a fact that cannot only be attributed to the ephemeral effect of the materials but also to the occlusion effect, as occlusion is known to boost phase I or the vascular event of an inflammatory response. Occurrence of phase II or the cellular event of the inflammatory process was not evidenced in our test result since there were no observable changes in the skin morphology. The toxicity test of SFKLRY-NH<sub>2</sub> also did not show any signs of irritation in the skin throughout the test period. This result is not only in agreement with our skin irritation test result in human volunteers, where the materials caused slightly barely perceptible erythema, but also may indicate that the degree of irritation is dose-dependent. This is because, while it was possible to see very slight erythema on the acute application of this materials in high concentration, there was no observable erythema in low concentration. The fact that neither erythema nor edema persisted in the skin could also lend support that the SFKLRY-NH<sub>2</sub>, synthetic peptide is safe to use at the indicated dose.

Among the known VEGF inducers such as TNF-α, transforming growth factor-β, interleukin-1β, and endothelin (Bussolino *et al.*, 1997), SFKLRY-NH<sub>2</sub> is the smallest peptide, which has several advantages over other proteins in the aspect of easier synthesis, lower cost than protein expres-

sion and with unnecessary expression system; we simply get high purity of peptides. Such a potential benefit of SFKLRY-NH<sub>2</sub> on vascular remodeling may suggest a potential use of SFKLRY-NH<sub>2</sub> for human disease evoked by an impaired blood supply, including foot and leg ulcers associated with diabetes or burn wounds.

In conclusion, no skin reaction was observed at 1 hour and 24 hour after removing these test materials in all human subjects. Therefore, we concluded that SFKLRY-NH<sub>2</sub> had minimal potential to elicit an irritation reaction. This is the first study performing all the toxicity tests on these materials for approval as external use for the skin application. We believe that SFKLRY-NH<sub>2</sub> can be safely utilized in cosmetic ingredients for human skin application.

## ACKNOWLEDGMENTS

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